Observations of the reproductive biology of *Acacia saligna* (Labill.) H.L. Wendl.

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Abstract

Acacia saligna (Labill.) H.L. Wendl. (subgenus Phyllodineae) has the potential to be developed as a biomass crop for the agricultural regions of southern Australia. This study examined the reproductive biology of *A. saligna* to facilitate the domestication and utilization of the species. The reproductive biology of *A. saligna* was found to be broadly similar to other Australia Acacia in the subgenus Phyllodineae. Flowering conformed to the phases described for other Acacia species. The flowers of *A. saligna* open asynchronously within a flower-head. Female sterility also was observed to occur in *A. saligna* with extent of sterility being highly variable. Mean reproductive output was found to be low. This work has identified a number of characters that will potentially make controlled crossing in *A. saligna* practically challenging. If a breeding program for the species is to be established then using open pollination techniques may be more practical and cost effective than controlled crossing.

Keywords: *Acacia saligna*, reproductive biology, floral biology, pollen quality, pollen storage and reproductive output.

Introduction

Acacia is a genus comprising more than 1,500 species, with the greatest diversity of species being native to Australia (Maslin *et al.* 2003). The majority of these species can be referred to the subgenus Phyllodineae (Maslin *et al.* 2003). Despite the large number of species in the genus the reproductive biology of only a limited number of species have been examined in detail (Kenrick 2003). The general characteristics of reproductive biology tend to be similar in all species of Acacia studied so far, though subtle differences between species have been identified (Kenrick 2003; Kenrick & Knox 1989; Sedgley 1989). Slight differences in reproductive biology can impact on utilization and management (Sedgley & Griffin 1989; Simmonds & Smartt 1999), so it will be necessary to examine the reproductive biology of all Acacia species of interest for utilisation in restoration and agroforestry.

A study that assessed the woody-crop potential of 462 Acacia native to the dryland cropping regions of southern Australia identified 35 species with good potential for domestication (Maslin & McDonald 2004). *Acacia saligna* (Labill.) H.L. Wendl. was ranked as one of the best of the 35 species because of its fast to very fast growth rates, ability to produce moderate to large amounts of biomass, coppicing potential in some genotypes, moderate to vigorous suckering, and potential to be cultivated over a reasonably wide geographic area. Acacia saligna is native to the south-west of Western Australia and is already planted extensively around the world.

Economically important traits, such as plant form, are known to be variable in *A. saligna* (Le Houerou 2002; Le Houerou & Pontanier 1987; Maslin *et al.* 1998; Nativ *et al.* 1999), and some of the variation may be the result of genetic diversity in the species, which suggests that the cultivation of improved cultivars could be possible (George *et al.* 2006). Development of improved cultivars via selection and breeding will enhance the value of the species as a crop plant. Breeding and cultivation of *A. saligna* will require information regarding the reproductive biology of the species (Sedgley 1996). This paper presents findings from an examination of the reproductive biology of *A. saligna* that will provide information useful for the both the domestication of the species.

Materials and methods

Acacia saligna exhibits considerable morphological variation. Four informal morphologically differentiated variants are currently recognized and at the time of writing the intention is to raise these to subspecies rank (Maslin in prep.). The subspecies are presently designated as *lindleyi*, *pruinescens*, *saligna* and *stolonifera*.

Study sites

A range of study sites were used in this research. The sites are listed in Table 1, together with latitude and longitude co-ordinates, information on subspecies present, and year sampled. The subspecies occupy separate but overlapping distributions in south-western Australia (Figure 1).

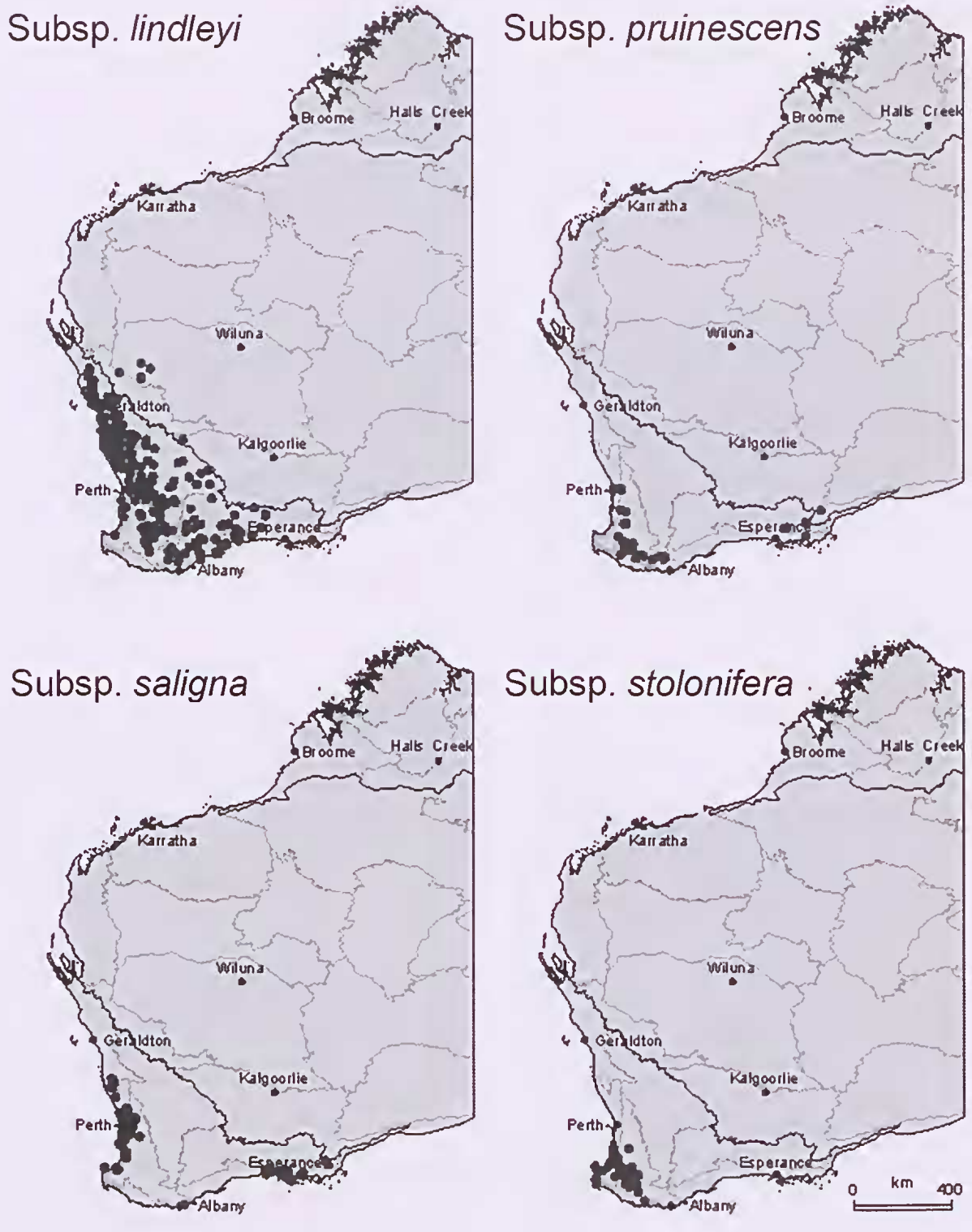

Figure 1. The ranges of the subspecies of *Acacia saligna* in south-western Australia (WAH 2009).

Table 1

Details of the sites used to assess reproductive output in *Acacia saligna*.

subspecies	Population Name	Year sampled	Lat. E	Long. S	WAHERB Sheet no.
saligna	Flynn	2005	31 41 14	115 46 03	6796273
saligna	Yunderup	2003/2005	32 34 20	115 47 27	6451411
saligna	Millar	2005	32 17 09	115 47 45	7285752
stolonifera	Hesters	2003/2005	33 54 19	116 06 22	6020232
stolonifera	Peppermint Gr	2003/2005	33 32 24	115 30 30	6020275
stolonifera	Leschenault	2003	33 13 04	115 41 36	6451438
stolonifera	Lowden	2005	33 32 32	115 56 51	7253176
pruinescens	Tweed Road	2003/2005	34 00 06	116 13 08	6020208
pruinescens	Tweed River	2005	34 00 21	116 29 31	6183336
pruinescens	Weinup	2005	34 15 29	116 271 5	7255144
lindleyi	Wickepin	2003/2005	32 37 48	117 23 01	6183174
lindleyi	Jilakin	2003	32 39 50	118 19 45	6183190
lindleyi	Moore River	2003/2005	31 03 54	115 33 10	6020283
lindleyi	Towerninning	2005	33 35 13	116 47 49	7252552

WAHERB Western Australian Herbarium

Floral biology

Aspects of floral biology examined in this study included temporal changes occurring during floral development (over daily and hourly timescales), the timing of stigmatic receptivity, and female sterility.

The progression of floral development was observed on trees of *A. saligna* subsp. *saligna*. Racemes with unopened flowers were tagged on six trees growing in the Matilda Bay Reserve. Individual flower heads within the racemes were observed every day at 10 a.m. for 12 days (flower head is used to refer to the globose collection of individual flowers). Attention was paid to the relative timing of the emergence of the style and stamens, pollen shed and the duration of individual flower heads.

Flowering events occurring on shorter time-scales were examined using the methods described by Sornsathapornkul & Owens (1998) and Tandon & Shivanna (2001). Whole branches were taken from six trees at the same reserve and 32 unopened but mature flower heads were collected from each branch, with 3 to 4 mm of peduncle left attached to each flower head. The flower heads were washed in distilled water to remove pollen, insects and other debris. Flower heads were then placed in Petri dishes containing agar (0.8% agar, 1% sucrose) by inserting the peduncle into the agar until the base of the flower head touched the agar media. The flower heads from a single tree were divided between two dishes with 16 heads per dish. The Petri dishes, with lids, were placed on a laboratory bench under fluorescent lighting. The temperature of the room was maintained at approximately 20°C throughout the experiment. Individual flower heads were observed via dissecting microscope every two hours for 32 hours. The number of flowers open within flower heads, the timing of the emergence of styles and stamens and the length of time taken for floral development were recorded.

The timing of stigmatic receptivity was determined using both enzymatic activity of stigmas and pollen tube growth. To assess enzymatic activity of stigmas, ten inflorescences were tagged on each of four trees of the subspecies saligna in the Matilda Bay Reserve. Flower heads were harvested at 10 a.m. at 0, 1, 2, 3 and 4 days after the first flowers in each flower head opened. The flower heads were immersed in 0.5 mg/ml α-naphthyl acetate in acetone, 0.1 M phosphate buffer, 2.5 mg/ml Fast Blue B Salt for 5 to 10 minutes. This stain indicates non-specific esterase activity, which is an indicator of stigmatic receptivity (Kenrick & Knox 1981). Mature and immature flower heads in which no flowers had opened were also sampled, dissected and stained.

To assess pollen tube growth, individual flowers from 16 mature flower heads were harvested from the receptacle as they opened, placed in agar plates and observed every 2 hours. Individual flowers were hand pollinated at 2, 10, 20 and 30 hours following anthesis. Before and after pollination the individual flowers were supported in agar plates (0.8% agar, 1% sucrose). Fresh pollen for pollination was collected by beating newly opened flowers on a clean microscope slide. The risk of selfing adversely affecting the results of this study was considered to be low because studies have shown no difference in pollen tube growth between selfing and outcrossing in *Acacia* (Kenrick *et al.* 1984). Despite this, to reduce the risk of genetically similar individuals affecting the results, trees used as a source of pollen were located approximately 1 km from the study population. To perform the cross, a single polyad was placed directly on the stigma of each flower under a dissecting microscope. Following pollination flowers were left for 12 hours and then fixed in glacial acetic acid:ethanol (1:2) at 5°C for 24 hours. To observe pollen tube growth in open pollinated flowers mature flower heads of *A. saligna* subsp. *saligna* were also harvested from the Matilda Bay Reserve populations and fixed.

A modified method of Kenrick & Knox (1985) was used to visualize pollen tubes. Flowers were soaked overnight in 8M NaOH and then stained using aniline blue (0.1% aniline, pH 4.5). Flowers were rinsed well in distilled water between each step. The prepared slides were kept at 5°C for 2 hours prior to viewing pollen

tubes using fluorescent microscopy. Pollen tubes were ranked on either their presence or absence and a qualitative assessment was made regarding the number of tubes per style and their length.

The presence of female-sterility was examined in eight wild populations of *A. saligna* in 2003. The populations examined were Yunderup (ssp. *saligna*); Hesters, Peppermint Gr. and Leschenault (ssp. *stolonifera*); Tweed Road (ssp. *pruinescens*); and Wickepin, Jilakin and Moore River (ssp. *lindleyi*) (Table 1). Three trees were sampled in each population. Six flower heads from each tree were dissected and examined under dissecting microscope. A flower was considered female-sterile if the gynoecium was absent or visibly malformed.

Pollen viability and pollen storage

To test the efficacy of different storage treatments in maintaining the viability of *A. saligna* pollen, flower heads were harvested from three trees at the Matilda Bay Reserve population, with 200 flower heads harvested per tree. Inflorescences were spread evenly on paper and left to air dry at 20°C for 4 hours before being bulked. Fine scissors were used to cut anthers from the inflorescence filaments to reduce the volume of material to be desiccated. The material from each tree was bulked and dried for 48 hours at either a) 5°C in a desiccator over a large volume of silica or b) in a HetoFD40 freeze drier at $1\times10^{-1.6}$ hPA. To prevent condensation following desiccation dried material was left in a desiccator and placed on the bench to warm to room temperature.

Samples of anthers were taken from the bulked material and put in Eppendorf tubes that were stored in zip-lock bags with ~30 g of silica. Bags from each treatment were then randomly selected and stored at either room temperature, 5°C, –20°C or –80°C. The viability of the pollen was tested at 9, 30, 59, 163 and 251 days. The viability of the pollen was assessed using the methods of Dafni (1992) and Sedgley and Harbard (1993). Flowers were placed in a 20% sucrose solution and agitated to release polyads. The suspension of polyads was left for 10 minutes to allow the grains to hydrate. A drop of 1% Fluorescein Diacetate (FDA) in acetone was evaporated on a microscope slide and a drop of the polyad-suspension added. The stained polyads were then viewed via fluorescence microscopy. Fluorescing grains were regarded as being viable. At least 50 polyads were scored for each sample and the relative numbers of viable polyads recorded. Due to the compound nature of *Acacia* pollen, viability was scored according to the method of Kenrick & Knox (1989) – if more that 50% of the grains within the polyad were fluorescing the grains was scored as being greater than fifty percent viable, and if less than 50% but more than 0% were fluorescing it was scored as being less than fifty percent viable. A score of zero viability was also applied if no grains were fluorescing.

Pod set

The pod set of *A. saligna* was assessed using similar methods to those described by Yates & Broadhurst (2002) and Gaol & Fox (2002). Examinations were carried out in the flowering seasons of 2003 and 2005. At each of the 14 populations listed in Table 1, 15 mature trees spaced at approximately 10 metre intervals were tagged. On each tree three 40 cm lengths of branch (measured from the apical tip and including lateral branches) were tagged. The branches were spaced throughout the flowering canopy. The number of open and unopened flower heads distal to each tag were counted. After pods had developed, but not fully matured and dehisced, the branchlets were collected and taken back to the laboratory and pod production was recorded.

Statistical analysis

All statistical analysis was carried out using the programs Genstat (Genstat 2002) and SPSS for Windows (SPSS 2008). Tests for significant differences between groups of normally distributed data were carried out using ANOVA and pairwise comparisons with Tukey's Least Significant Difference (Genstat 2002). Data regarding female sterility and reproductive output was found to be non-normally distributed. The closest approximation for these data sets is an exponential distribution so a statistical method for comparing exponential distributions was used to test for significance between the subspecies means (Weerahandi 2003).

Results

Floral biology

In wild trees, individual flowers in a flower head were observed to open asynchronously, and flower heads within inflorescences were also observed to open asynchronously, usually with basal flower heads opening first. Opening of flowers within an inflorescence occurred over a period of 1 to 2 days. Once all the flowers were open, the flower head persisted for 5 to 7 days until the individual flowers began to detach from the receptacle. The pistil and stamen appeared to emerge either synchronously or in quick succession. When the flowers in a flower head were fully open, the style was typically around 1 mm longer than the stamens. Pollen release occurred over a period of 1 to 2 days after the flowers opened.

Observations of floral opening in the laboratory showed the first event was splitting of the perianth in individual flowers (Figure 2A and B) which was then followed by the emergence of a 'tuft' of anthers 1 to 2 mm in length (Figure 3A). Following the emergence of the anther-tuft the style elongated, followed shortly by elongation of stamens (Figure 3B and C). The non-elongated style was folded inside the flower bud (Figure 4). The opening of the flowers in a flower head was asynchronous, the basal flowers were typically the first to open. Throughout the process of floral opening the style typically remained 1 to 2 mm longer than the stamens (Figure 5A and B). In 12% of observations the fully extended stamens elongated to a length comparable with the styles.

The time period between perianth splitting and emergence of the anther-tuft was between 2 and 14 hours, but typically less than 8 hours (Figure 6A). The style emerged before the stamens elongated in 59% of cases, whereas both the style and stamens emerged together in 41% of cases. When the style emerged alone, it reached a length of 1 to 2 mm before the stamens began to elongate, which was typically 2 to 8 hours later (Figure 6B). The remaining flowers in a head took an average of

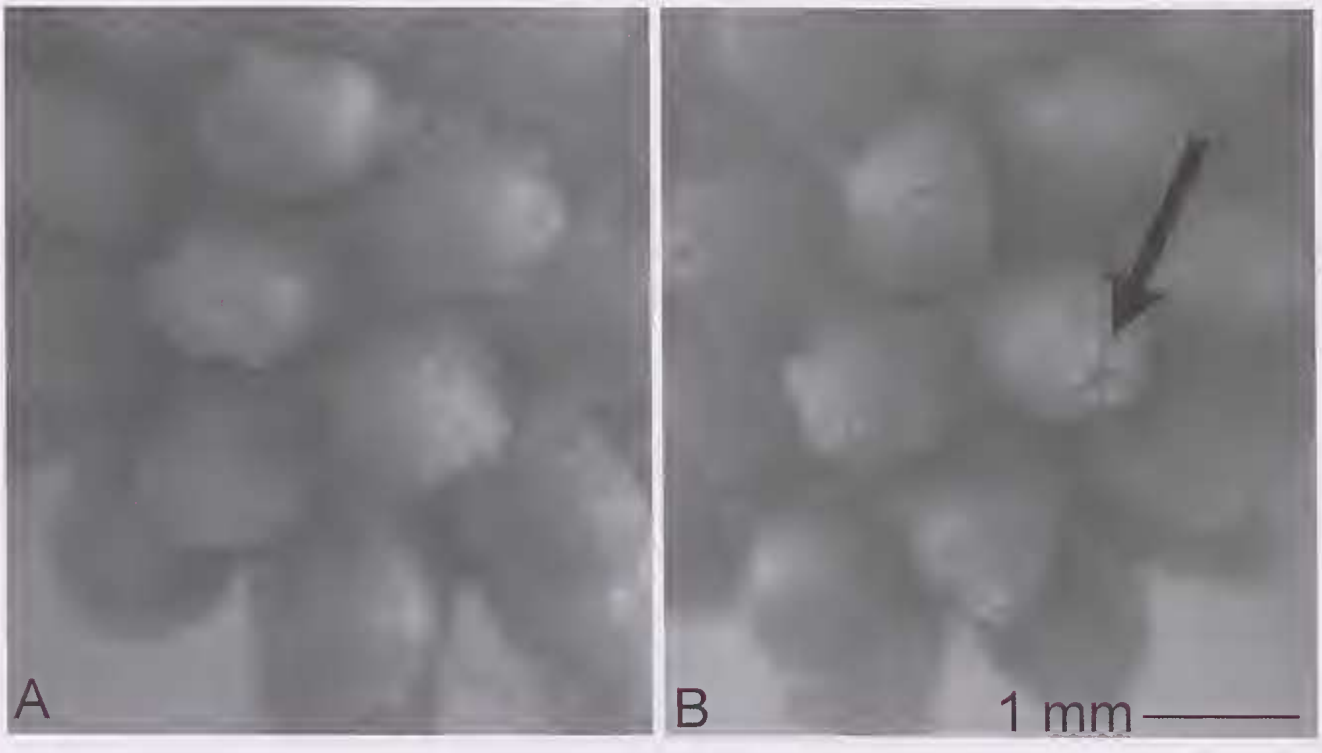

Figure 2. An inflorescence of *Acacia saligna* showing (A) unsplit perianth and (B) split perianth (arrowed).

Figure 3. Individual flowers of *Acacia saligna* showing sequentially the (A) newly opened flower with stamen 'tuft', (B) elongation of style, and (C) elongation of stamens.

Figure 4. A gynoecium from unopened flower, showing folding of style.

9 hours to open, with a standard deviation of 5 hours (Figure 6C).

Drops of fluid were observed on the stigma of some flowers between 10 and 26 hours following opening. In addition, no pollen shed was observed in any of the flowers in the laboratory. The samples were in closed Petri dishes so it was suspected that failure to shed pollen due to humidity. Upon completion of the laboratory observations the tops were removed from the Petri dishes and as the flowers were allowed to air-dry pollen-shed was observed.

Enzymatic activity, as indicated by staining, was not observed in stigma from immature buds (Figure 7A). Stigma from mature unopened-buds, and flowers open for 1, 2, 3, and 4 days, exhibited enzyme activity (Figure 7B and C). Some stigma with pollen grains present failed to show enzymatic activity (Figure 7D). Only a single pollen grain was observed on each stigma (Figure 7C and 7D).

Figure 6. Observations of the time of the opening of flowers of *Acacia saligna*. (A) Time between the splitting of the perianth and the emergence of stamens, (B) Time (hrs) between the emergence of the style and the emergence of stamens. (C) The initial number of flowers to open in each head.

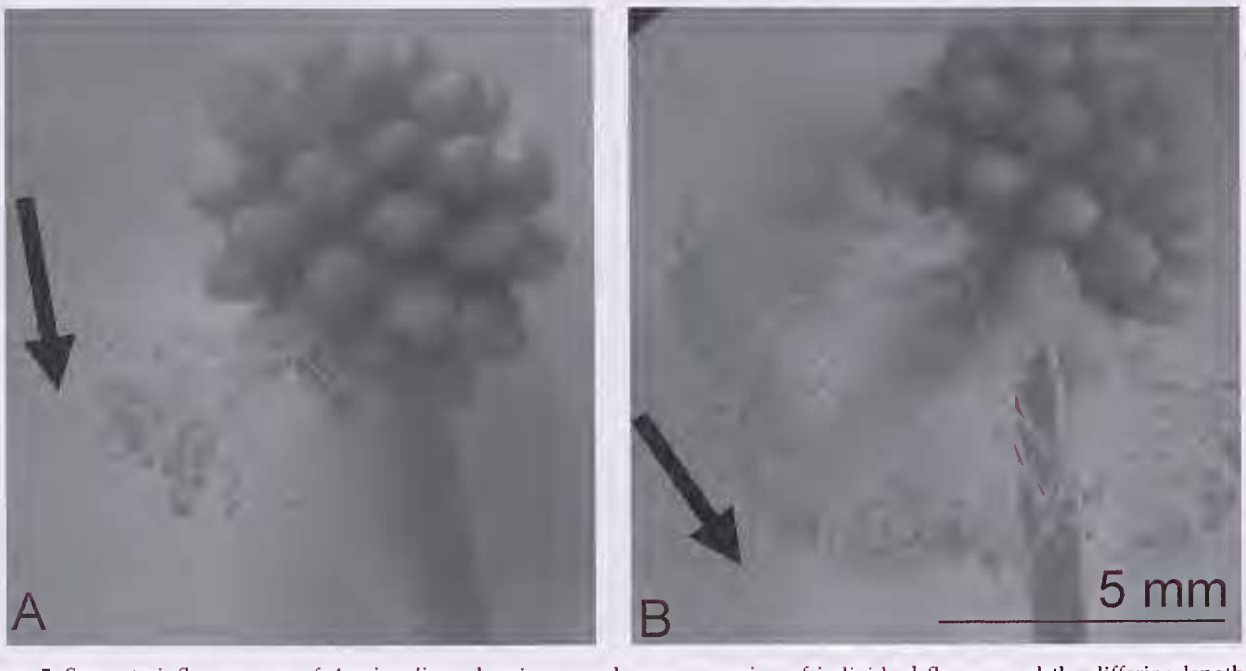

Figure 5. Separate inflorescences of *Acacia saligna* showing asynchronous opening of individual flowers and the differing lengths of styles and stamens (arrowed).

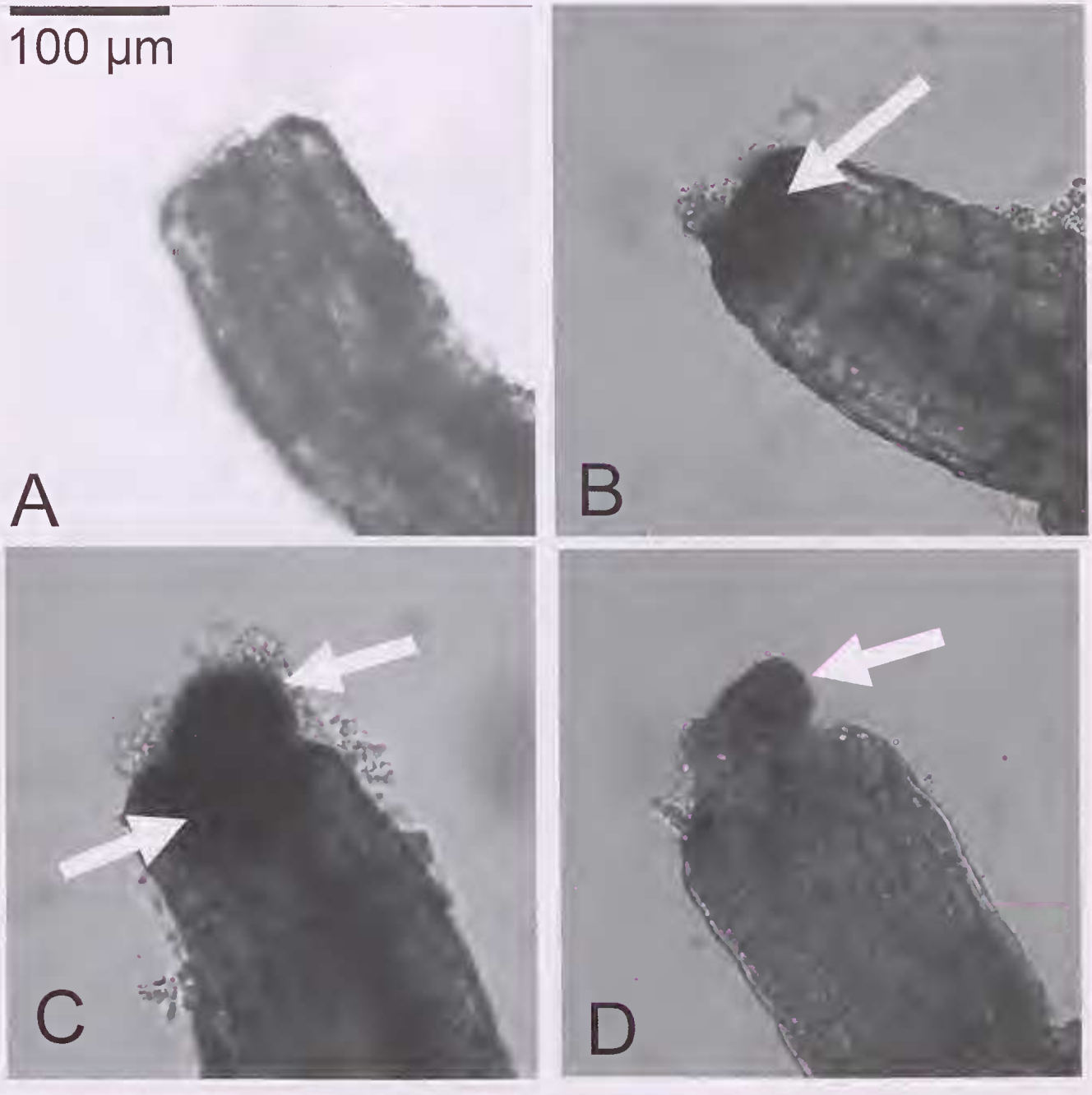

Figure 7. Stigma of *Acacia saligna* stained using á-naphthyl acetate and fast blue B salt to indicate esterase activity. Esterase activity indicates potential stigmatic receptivity. (A) A stigma from an immature bud showing no enzyme activity. (B) A stigma from a mature unopened flower showing enzyme activity (arrowed), the stigma of mature but unopened flowers show similar activity. (C) Stigma with pollen grain (arrowed). (D) Stigma with pollen grain showing no staining reaction.

Pollen tube growth was observed in flowers pollinated by hand in the laboratory. Pollen tubes were observed in the styles of flowers pollinated 2 and 10 hours after anthesis, although none of the tubes had reached the ovules. Pollen tubes were also observed in the styles of flowers pollinated 20 and 30 hours after anthesis. Some pollen tubes had reached the ovules in these flowers. Exudate was present on the stigmas of 10, 20 and 30 hour old flowers prior to hand pollination, and was inevitably blotted off when the polyads were applied. Exudate was seen to return following pollination. This pre-pollination exudate was not observed on 2 hour old flowers but exudate was observed following pollination in these flowers.

Female sterility was present in all subspecies of *A. saligna* as reduced gynoecia that were orange in colour (Figure 8A and B). The character was highly variable, with the mean value for female sterility across all populations being 10 % with a standard deviation of 17 %. The maximum number of female-sterile flowers in a flower head was 96%. There was no significant difference in the occurrence of female sterility between populations or between subspecies.

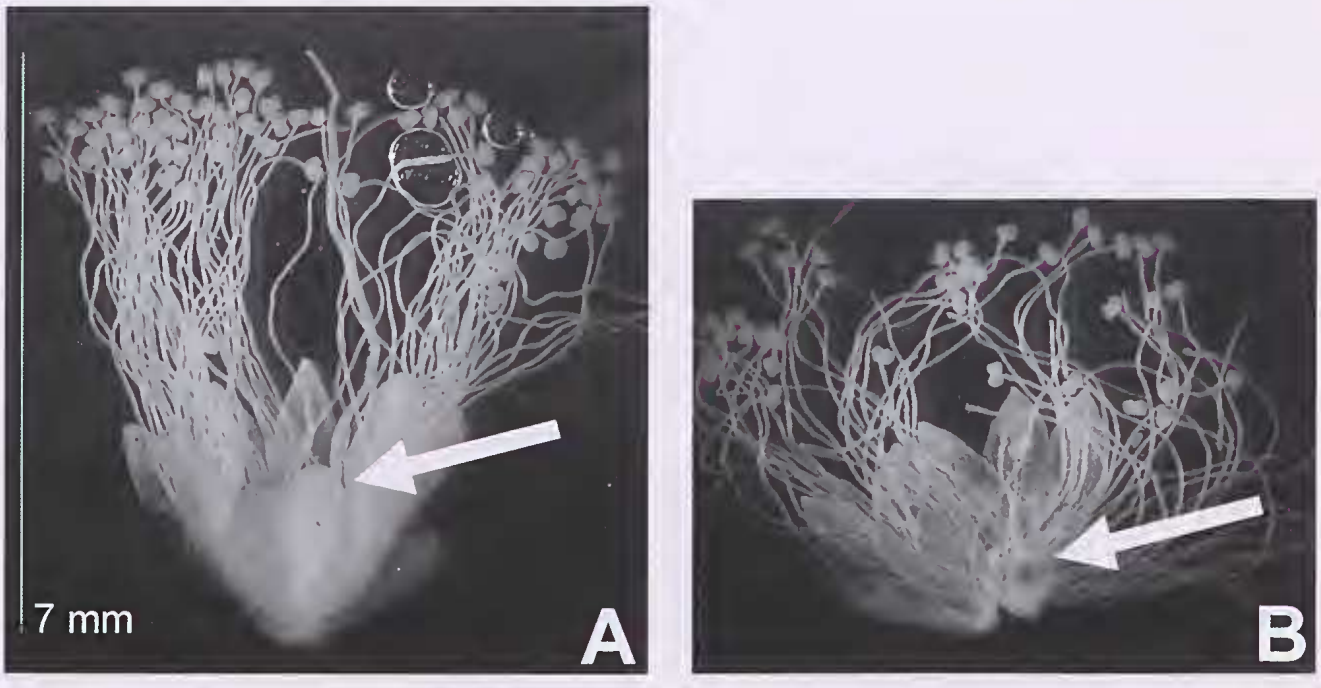

Figure 8. Individual flowers of *Acacia saligna* showing (A) a hermaphroditic flower with a normal gynoecium (arrowed) and (B) a female sterile flower, with reduced gynoecium (arrowed).

Pollen viability and pollen storage

Initial tests showed that pollen stored without being desiccated quickly lost viability. The viability of undesiccated pollen decreased to 0% after eight days stored at room temperature. When kept at –20°C 4% of the polyads retained >50% viability after 33 days, but this had decreased to 0% after 61 days. When kept at –80°C 2% of the polyads had retained >50% after 153 days. Based on these findings a non-desiccated treatment was not pursued in further experiments.

The viability of desiccated pollen was found to decrease to 0% after 163 days when stored at room temperature but retained some viability after 251 days when stored at 5°C, –20°C and –80°C (Figure 9). After 251 days storage, the average number of polyads with >50%viablity was between 0–5%. The number of polyads with <50% viability was also low but there were significant differences between storage temperatures and desiccation methods. The samples desiccated at 5°C using silica gel and then stored at low temperatures had significantly higher viability than those stored at room temperature ($p<0.01$)). The mean viability of pollen desiccated using a vacuum was lower than the pollen desiccated using silica however the difference was not significant.

Pod set

The pod set of *A. saligna* is summarized in Table 2. The pod set was found to differ significantly between the subspecies. The ssp. *pruinescens* and *saligna* had significantly higher pod set than the other subspecies and did not differ significantly from each other, whilst ssp. *stolonifera* and *lindleyi* both differed significantly from each other.

Table 2

A summary of the pod set of *Acacia saligna*. Commonality of letters indicates non-significant difference between means at the 5% level. Standard deviation in parentheses.

Subspecies	Pod set
Overall	$0.08^{(0.13)}$
lindleyi	$0.05^{(0.10)a}$
stolonifera	$0.08^{(0.14)b}$
saligna	$0.11^{(0.14)c}$
pruinescens	$0.12^{(0.15)c}$

Discussion

The reproductive biology of the various subspecies of *A. saligna* was found to be broadly similar to other Australian *Acacia* species. The flowering phases observed in ssp. *saligna* generally conform with the phases of flowering described for other *Acacia* species by Knox *et al.* (1989), being protogynous, with the style emerging before the stamens. The apparent concurrent emergence of the style and stamens in some instances may be an artefact of the 2 hour time period between observations.

The stigma of ssp. *saligna* were found to be receptive to pollen upon emergence, as has been observed in other *Acacia* (Kenrick 2003). This may represent a brief female-phase which lasts a few hours prior to the elongation of the stamens. Generally though, the female and male phases appear to overlap, with the stigma remaining receptive to pollen for at least 30 hours after emerging, while all the stamens had elongated and begun to shed pollen.

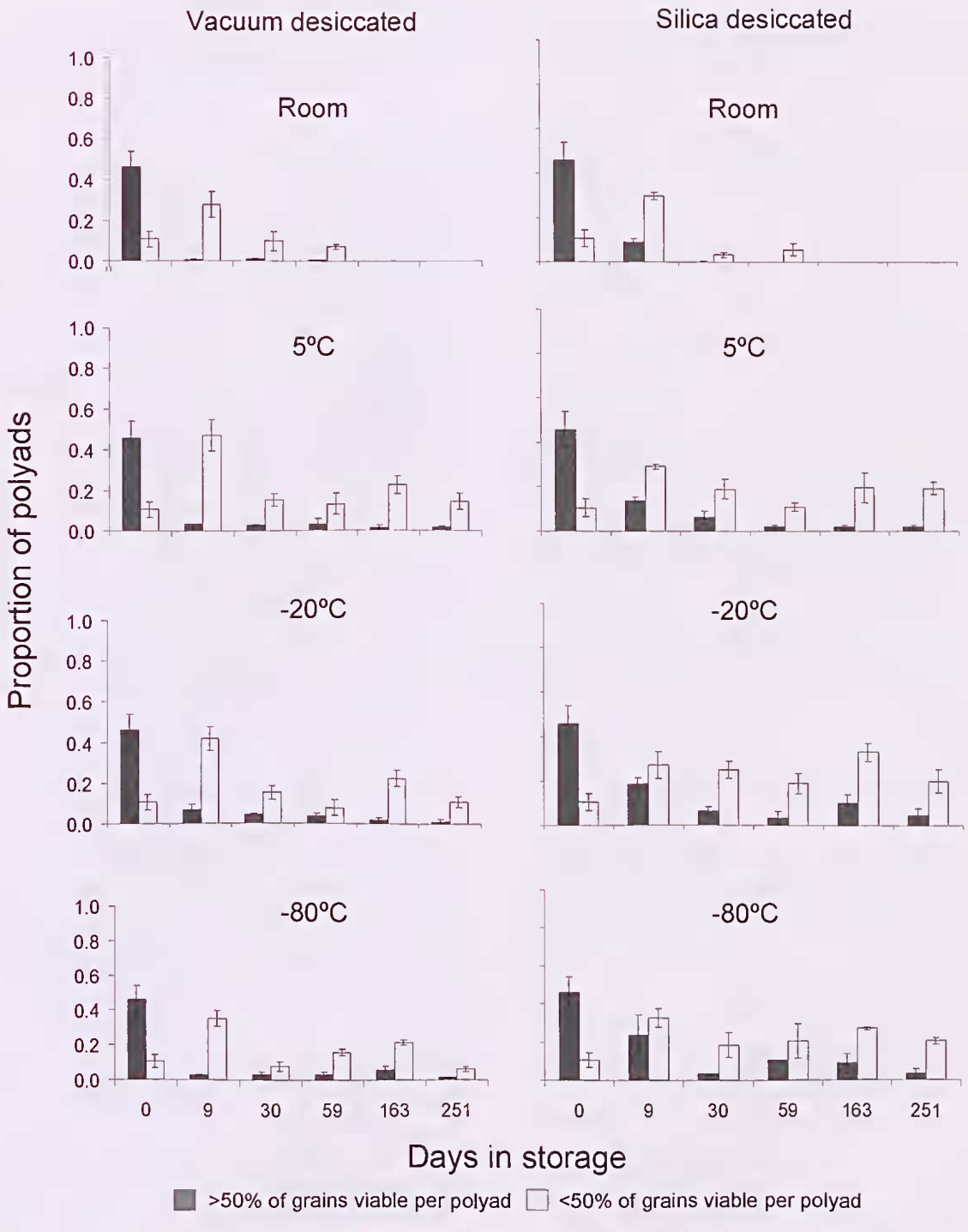

Figure 9. The viability of pollen of *Acacia saligna* following storage at different temperatures. Standard deviation is indicated.

The timing of the male phase is unclear because pollen shed appears to be mediated by ambient humidity levels. This has also been observed in other *Acacia* (Stone *et al.* 2003; Stone *et al.* 1998). Some pollen shed may be possible only a few hours following the emergence of stamens provided conditions are dry, however peak pollen shed appears to occur 24 hours after the stamens emerge.

The length of the female and male phase, and duration of the flowers, in ssp. *saligna* is within the range seen in other Australian Acacia where the female phase ranges from 1 to 10 days in length, male phase from 1 to 8 days, and flower head longevity varies between 4 and 12 days (Stone *et al.* 2003; Stone *et al.* 1998).

Acacia species within Phyllodineae show both synchronicity and asynchronicity in floral opening within a head. Asynchronicity is hypothesized to be an adaptation favouring smaller pollen vectors (Kenrick 2003; Stone *et al.* 2003). Given that flowers of subspecies saligna open asynchronously this suggests that small insects may be the primary pollen vector.

Female sterility within a flower head was observed to occur in all subspecies of *A. saligna* at a mean rate of 10 %

of flowers within a flower head. Female sterility has been observed in other species of *Acacia* as a highly variable and plastic character influenced by the environmental conditions experienced by the plants during floral development (Kenrick 2003; Sedgley 1989). There is insufficient information regarding environmental conditions at the study sites to attribute the variability in female sterility to any specific causes.

The pod set of *A. saligna* was low overall and showed significant differences between subspecies. This low pod set is comparable to results obtained by another study of the pod set of *A. saligna* conducted in the wheatbelt region of south-western Australia that found the proportion of inflorescences to set pods to be between 5.5 to 12.5% (Gaol & Fox 2002).

The domestication of *A. saligna* will necessarily involve the selection and cultivation of improved cultivars of the species. This work has identified a number of characters that will potentially make controlled crossing in *A. saligna* practically challenging, notably, the flower heads of *A. saligna* are small and dense, flowering is asynchronous, and it is difficult to maintain the viability of pollen in storage. Furthermore, compound pollen grains, of variable viability, and comparatively low seed-set mean that controlled crossing will provide low returns. The findings of this work suggest that using open pollination techniques may be more practical and cost effective than controlled crossing. The study has also shown that differences in certain aspects of reproductive biology may exist between the subspecies of *A. saligna*, particularly in terms of reproductive output. More work will be needed to explore these differences.

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